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Specificity of the volume-sensitive sodium pump inhibitor isolated from human peritoneal dialysate in chronic renal failure

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Specificity of the volume-sensitive sodium pump inhibitor isolated from human peritoneal dialysate in chronic renal failure. Compromised renal function predisposes to volume-dependent hypertension. Increased plasma levels of a sodium pump inhibitor as a possible pathogenetic factor have been demonstrated by many investigators in such patients, but efforts to identify the responsible agent have led to many, diverse candidates. Our premise in this study is that candidacy must depend on the satisfaction of rigorous criteria, including a specific action of the agent on the sodium pump. These criteria included reversibility, concentration dependence, receptor mediation, and an action at the appropriate step in the enzyme cycle. These criteria were applied to a potent [Na,K]ATPase inhibitor we have identified in the peritoneal dialysate of patients with chronic renal failure, present only during extracellular fluid volume expansion, the levels of which are correlated with the blood pressure rise that results from excessive NaCl and water intake. In microsomes that contained both [Na,K]ATPase and other ATPases, this candidate inhibited only the Na and K dependent, ouabain-sensitive ATPase. It displaced ouabain from the cardioglycoside binding site and its binding was linked to inhibition. Inhibition was produced by slowing the pump's dephosphorylation step, the exact action of all cardioglycosides. Finally, the candidate cross reacted with a digoxin Fab fragment and this Fab reversed its inhibition of [Na,K]ATPase. Together, these experiments demonstrate that the PD candidate specifically, and reversibly, inhibits the sodium pump via the cardioglycoside binding site, and hence, meets this crucial criterion for candidacy.

As renal disease compromises kidney function, a volume dependent form of hypertension commonly arises [1, 2]. Although the pathogenesis of this hypertension is probably complex, many investigators have demonstrated in such patients a circulating inhibitor of the sodium pump, which is capable of causing vasoconstriction and would appear to link volume status to blood pressure [3–13]. Studies in animal models of salt sensitive hypertenstion have consistently produced similar findings [14–18]. Despite repeated and consistent demonstrations of a correlation between renal function, volume status, sodium pump inhibitory activity and blood pressure, hypotheses related to this inhibitor have not achieved universal acceptance. The controversy continues, in part, for at least two reasons: First, patients with reduced renal function accumulate a wide variety of compounds that may not be relevant. Second, many diverse compounds have been claimed as the responsible factor, often supported by a very limited range of scientific criteria, creating confusion and skepticism [19–22]. Indeed, a wide range of physiologic compounds when present at high concentrations may affect [Na,K]ATPase activity, but act in a non-specific manner. Examples would include agents that denature the enzyme or modulate its activity by altering the membrane environment or that broadly modify peptides.

For a factor to be considered a pathogenetic candidate, its influence on the sodium pump should demonstrate reversible, concentration-dependent inhibition acting via the cardioglycoside binding site [20]. This study was designed to evaluate the biochemical and immunological specificity of a potent inhibitor found in the peritoneal dialysate of renal failure patients, in close correlation with volume status and blood pressure [23]. Our first criterion in this study, as previously described [23], was that the responsible agent had to appear during volume expansion, and in association with a rise in blood pressure. In this study, we have applied a second criterion, a crucial step in the validation of the candidacy of the agent. As opposed to the nonspecific actions described above, a specific [Na,K]ATPase inhibitor must induce reversible, concentration-dependent, receptor-mediated inhibition at the appropriate step in the enzyme cycle. Such an agent would act on the cardiac glycoside or digitalis binding site. We tested three hypotheses: (i) The effects of the PD candidate are reversible. (ii) The PD candidate inhibits [Na,K]ATPase by an interaction with the cardioglycoside binding site and specifically inhibits the pump cycle by interrupting the desphosphorylation step, analogous to all known cardioglycosides. (iii) The PD candidate acts exclusively on [Na,K]ATPase and not on other ATPases. We employed several assays to test each of these hypotheses, and included ouabain and other cardioglycosides as specific inhibitors as well, and included non-specific inhibitors, specifically oleic acid and lysophosphatidyl choline. The results indicate that the volume-sensitive candidate isolated from human peritoneal dialysate acts specifically and reversibly on the sodium pump, via the digitalis binding site.

Methods

PD candidate acquisition and purification

As described in detail earlier [23], our studies employed peritoneal dialysate (PD) from patients with chronic renal failure, who were maintained on chronic peritoneal dialysis, as a source for the sodium pump inhibitor. When NaCl and water intake were

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increased for four days, the accompanying volume expansion, evidenced in a 3 to 9 kg weight gain, was associated with a rise in blood pressure and the appearance in plasma and in peritoneal dialysate of a sodium pump inhibitor that was not present during volume contraction. On processing PD [23–25], only one HPLC fraction at 19.5 ± 0.5 minutes elution showed an increase in [Na,K]ATPase activity with volume shift. All of the studies on chemical specificity that follow have been performed with this factor.

For these studies, ~2 liter PD from volume expanded individuals was collected fresh the morning of each experiment. A detailed description of its purification is provided elsewhere [23]. In brief, the dialysate was ultrafiltered through a 1000 dalton exclusion membrane under argon. The active factor was removed from the filtrate and volume reduced by solid phase extraction using a preparative C_{18} reversed phase HPLC guard column. After elution with 100% methanol and drying, the sodium pump inhibitor was submitted to two C_{18} HPLC gradient fractionations. The location of the fraction of interest was previously established by several criteria [23]. Its occurrence at 19.5 ± 0.5 minutes elution allowed for it to be distinguished from other inhibitors of [Na,K]ATPase, and hence it is referred to as the PD candidate. On completion of this purification the PD candidate was used immediately. The amount of PD candidate thus produced varied significantly with patient, patient state, chromatographic losses (which were significant), and decomposition. Typically the product of one purification was divided, from 1/5 to 1/15 being used in a single reaction, representing 100- to 3500-fold increase in concentration in the absence of any loss. Ouabain and digoxin standards applied to this same HPLC system eluted at 13.5 and 23.0 minutes, respectively, and would have been completely resolved from the PD candidate if present in PD.

Preparation of [Na,K]ATPase

[Na,K]ATPase was purified from calf kidney outer medulla as described previously [24, 25]. Briefly, microsomes were produced by homogenization of dissected medullary tissue followed by two different centrifugations (10,000 × g, 30 min and 48,000 × g, 45 min). The pellet representing the microsomal [Na,K]ATPase was further purified by addition of 1.4 mg/ml deoxycholate, followed by ultracentrifugation (180,000 × g, 110 min) through a two-layer discontinuous gradient of 23% and 38% sucrose. The activity resided at the interface of the 38% and the 23% sucrose fractions. All experiments involving [Na,K]ATPase used enzyme prepared in this manner unless otherwise noted.

Tests of the reversibility of the PD candidate's actions

Cross reactivity with digoxin antisera. Physiologic suppression of enzyme activity is reversible, that is, does not irreversibly denature or poison the enzyme. In the absence of a specific antagonist of the PD candidate or cardioglycosides, the most compelling alternative approach is to reverse inhibition by antibody binding of the inhibitor (immunoneutralization) with a restoration of activity. To accomplish such experiments, the first requirement was to identify antibodies with affinity for the PD candidate and establish a relationship between crossreactivity and biochemical activity. Initially PD candidate cross reactivity was assessed using a digoxin RIA (components obtained from Dupont-NEN, Billerica, MA, USA) described in detail previously [26, 27]. Purified specimens of PD candidate from peritoneal dialysate were collected, dried, resuspended in 10 mmol/liter sodium phosphate (pH = 7.4) and then divided among two dilution sets. The PD candidate available for one assay was diluted 1/2, 1/4, 1/10, 1/20, and 1/40. One set was assayed in the [Na,K]ATPase assay described above and the other in the digoxin RIA.

In a second set of experiments digoxin, the PD candidate, ouabain, strophanthidin and bufalin were serially diluted and assayed in duplicate in the same digoxin RIA. Lysophosphatidyl choline (LPC), a non-specific sodium pump inhibitor, at concentrations of 10^{-3} and 10^{-2} mol/liter was also assayed. Data are expressed as the fraction of labeled digoxin bound to antibody for each concentration of analyte (B) divided by the fraction of labeled digoxin bound in the absence of analyte (B_o) and plotted as the ratio of B/B_o versus the concentration.

Immunoneutralization with digoxin antibody Fab fragment. The previous experiments demonstrated an interaction between digoxin antibodies and digoxin, ouabain, strophanthidin, bufalin and the PD candidate. We then set out to extend this observation to a digoxin antibody Fab fragment that has been effective in a volume sensitive model [7]. In the first set of experiments employing the Fab fragment, we sought to prevent an inhibitory action by immunoneutralization. In these experiments, 20 μl of the PD candidate ($N = 6$), dried and resuspended in 100 μl sodium phosphate buffer (pH 7.4), ouabain (5×10^{-7} mol/liter, $N = 9$), bufalin (1×10^{-7} mol/liter, $N = 6$) and LPC (7×10^{-5} mol/liter, $N = 4$, as a non-specific inhibitor and control) were incubated with 2×10^{-6} mol/liter Fab fragments of an anti-digoxin antibody (Digibind, Burroughs-Wellcome, Triangle Park, NC, USA; FW ~50,000) or with 1×10^{-6} mol/liter sheep IgG in 20 μl sodium phosphate buffer for one hour at 37°C. After the incubation, 100 μl of buffer A [(in mmol/liter) 100 Na, 5 K, 3 Mg and 80 Tris (pH 7.4)] containing [γ-^{32}P]ATP (final concentration 4 mmol/liter), and 10 μl of [Na,K]ATPase were added directly and incubated for 30 minutes at 37°C to measure ATPase hydrolytic activity. The workup thereafter was identical to the [Na,K]ATPase assay described below.

The second series of experiments examined the ability of the digoxin Fab fragment to reverse the action of the PD candidate. The inhibitor (PD candidate, $N = 8$) was divided into four equal portions and each allowed to incubate with the enzyme for 30 minutes. To two of these incubations, the digoxin Fab fragment was introduced (1×10^{-6} mol/liter) to determine whether the Fab could reverse the effects of the PD candidate. In parallel experiments, paired aliquots of ouabain (1×10^{-7} mol/liter, $N = 6$) and lysophosphatidyl choline (7×10^{-5} mol/liter, LPC; $N = 6$), included as a non-specific inhibitor control, were followed by addition of 1×10^{-6} mol/liter Fab fragments to one of each pair. The reaction for all aliquots was then started by adding [γ-^{32}P]ATP and followed the method described for the [Na,K]-ATPase reaction. Several concentrations of ouabain were used and compared to the mean of the PD candidate response.

Tests of the PD candidate acting specifically via the digitalis binding site

[Na,K]ATPase assay. This assay is the one most commonly employed by investigators to assess the PD candidate activity. Inhibition of [Na,K]ATPase hydrolysis is a necessary but insufficient feature of any specific Na-pump inhibitor, in as much as non-specific inhibitors (or enzyme inactivators) are common, especially when purified [Na,K]ATPase is used [19–22, 25]. While

a useful and important test, the assay does not assess whether an inhibitor binds to the digitalis binding site. The activity of [Na,K]ATPase and its inhibition were determined by measuring the hydrolysis of [γ-^{32}P]ATP. Twenty microliter aliquots of PD candidate from a total of 300 μl or ouabain were incubated with 10 μg of enzyme for 30 minutes at 37°C in 110 μl of Buffer A or as specified in legends to the Figures. The hydrolysis was started by adding 10 μl of 40 mmol/liter [^{32}P]ATP (final concentration 4 mmol/liter ATP) and ended after 30 minutes as described above. Ouabain-insensitive activity was defined as the activity remaining in the presence of 1 mmol/liter ouabain. Specimens were typically assayed in duplicate.

Ouabain binding. The ability of compounds to inhibit ouabain binding to [Na,K]ATPase has also been frequently used to assay DLF. Binding to and competition for the digitalis binding site, is an essential feature of all cardioglycosides and hence applicable to candidates for a specific inhibitor of [Na,K]ATPase. While this activity is an important requirement, it is not adequate. Some compounds cause non-competitive, non-specific displacement of labeled ouabain through more generalized effects on the enzyme, such as an influence on the binding site microenvironment or on protein conformation. Binding of [^{3}H]ouabain to [Na,K]ATPase was measured in the presence of Mg and Pi, which selectively enhance binding of inhibitors acting via the digitalis receptor. The enzyme (15 μg) was incubated with $\sim 1 \times 10^{-8}$ mol/liter [^{3}H]ouabain and 40 μl of unlabeled ouabain or PD candidate (of 300 μl total) to 200 μl of a solution containing (in mmol/liter): 5 Mg, 5 Pi and 50 Tris (pH 7.2) for one hour at 37°C. The free ouabain was separated from bound ouabain by centrifugation at 290,000 × g for one hour. In parallel experiments the nonspecific binding, determined in the presence of 1 mmol/liter cold ouabain, was less than 5% of total radioactivity bound to the enzyme, and was subtracted. The relatively low specific activity of the [^{3}H]ouabain commercially available necessitated that relatively high concentrations of ouabain were required to maintain adequate counts of radioactivity. Typically the specimens were assayed in duplicate.

Phosphorylation. The enzyme cycle of the sodium pump includes an autophosphorylation step [28]. Cardioglycosides inhibit the pump cycle via a reduction in the dephosphorylation rate of this phosphoenzyme species (E-P) resulting in increased levels of phosphoenzyme with cardioglycoside inhibition. This assay is much less sensitive to non-specific inhibition of enzyme activity and requires that a candidate operate in a fashion equivalent to all known cardioglycosides [28]. Some consider it to be the most specific test for a specific pump inhibitor [19, 28]. Phosphorylation assays were carried out at 22°C for 20 seconds in the absence or presence of inhibitor and a reaction volume of 100 μl containing 10 μg [Na,K]ATPase and (mmol/liter): 3 Mg, 150 Na, 30 imidazole (pH 7.4) adding ~2 nmol/liter [γ-^{32}P]ATP to start the reaction. The reaction was then terminated 20 seconds later by adding 0.5 ml of an ice-cold stopping-solution containing 10% trichloroacetic acid, 1 mmol/liter ATP and 10 mmol/liter pyrophosphate. The mixture was allowed to stand for 10 minutes on ice before centrifugation. The protein was separated by centrifugation and washed once with 0.5 ml ice-cold 10% trichloroacetic acid. The precipitate was dissolved in 0.5 ml of 0.5% SDS and the radioactivity was counted. Nonspecific binding of [^{32}P]Pi was obtained by addition of medium without Mg and Na and was subtracted from total counts of radioactivity. For these experiments buffer alone ($N = 6$), ouabain at concentrations of 10^{-7} ($N = 4$), 10^{-6} ($N = 6$), and 10^{-5} mol/liter ($N = 4$), and PD candidate ($N = 5$) were tested.

Dephosphorylation. After phosphorylation of enzyme for 10 seconds on ice in the absence of inhibitor, the reaction was quenched by adding 100 μl of a solution containing 1 mmol/liter non-radioactive ATP and PD candidate ($N = 6$) or ouabain ($N = 6$) at each of three concentrations (10^{-5}, 10^{-6}, and 10^{-7} mol/liter or continued absence of inhibitor (as the control), and dephosphorylation was allowed to occur for 10 seconds (at 37°C) until the reaction was terminated with the stopping-solution employed for the phosphorylation assay, and the phosphoenzyme level was determined as described above.

Tests of PD candidate specificity to [Na,K]ATPase

Inhibition of ouabain sensitive and insensitive [Na,K]ATPase activity. Cardioglycosides exert their effects on only one enzyme, the sodium and potassium-stimulated [Na,K]ATPase. Other ATPases do not bind cardioglycosides nor is their activity modulated by them. Hence, a simple, yet important, requirement of any candidate for the endogenous Na-pump inhibitor is that its inhibition be limited to [Na,K]ATPase. We used two approaches to assess this requirement. The first took advantage of the observation that crude calf, kidney microsomes contained both ouabain-sensitive and insensitive ATPase activity.

Microsomes were prepared as described above. To 10 μg of microsomes in a buffer A, a maximal inhibitory dose of ouabain (1×10^{-3} mol/liter) was incubated for 30 minutes at 37°C and compared to an identical incubation omitting the ouabain. Thereafter, 10 μl of a 40 mmol/liter [γ-^{32}P]ATP solution (Amersham, Arlington Heights, IL, USA; final specific activity ~70 mCi/mol) was added to each reaction and the hydrolysis allowed to occur for an additional 30 minutes to determine the portion of ATPase activity that was ouabain-sensitive, and hence represented [Na,K]ATPase. After the incubation the reaction was terminated by adding a known volume of a 4% charcoal/0.1 N HCl solution. After mixing and centrifugation (10 min at 1200 × g), a known aliquot of supernatant was removed and radioactivity counted. In an attempt to identify the substrate and nature of the other ATPase activity, in separate aliquots, thapsigargin (3.3×10^{-5} mol/liter), an inhibitor of microsomal Ca-ATPase [29] was added to microsomes in the presence or absence of 1×10^{-3} mol/liter ouabain in an identical fashion. Finally, other aliquots of ouabain at concentrations causing intermediate levels of [Na,K]ATPase inhibition (2.5×10^{-5} mol/liter) or maximal inhibition (1×10^{-3} mol/liter) were incubated in the presence or absence of PD candidate. The inhibition produced by the other half of the PD candidate preparation by itself was simultaneously measured.

We also performed other experiments with oleic acid, a known non-specific inhibitor of ATPase activity, as a negative control for specific action. Oleic acid at concentrations of 2.5×10^{-5} and 1×10^{-4} mol/liter was incubated with and without 1×10^{-3} mol/liter ouabain and inhibition of ATPase activity assessed.

Inhibition of Na and K stimulated ATPase activity. The second approach to insure specificity used the same crude microsomes but now assessed [Na,K]ATPase activity by omitting Na and K, which are requisite for its activity but are not required for other ATPases. Specifically, 10 μg of crude microsomes were incubated with ouabain (1×10^{-7} mol/liter, $N = 6$) or with divided preparations of PD candidate ($N = 6$), half in buffer A and half in buffer A omitting the Na and K. The procedure described above

was followed thereafter. Oleic acid (2.5×10^{-5} mol/liter, $N = 6$, as a non-specific ATPase inhibitor, was tested in like fashion.

Statistical analysis

Data are expressed as the mean ± the SEM. Comparisons between one set of conditions and another for a single agent being tested were statistically tested using Student's *t*-test. Comparisons of several observations to a control were made by ANOVA, with post-hoc, pair-wise comparisons using Dunnett's test. Comparisons of two assays employing paired observations were made by Pearson's Product Moment Correlation test. A P-value <0.05 was considered significant.

Results

Preparation of a volume-sensitive sodium pump inhibitor from human peritoneal dialysate

The detailed studies that follow assessed a single sodium pump inhibitor from PD, the activity of which increased with volume expansion in renal failure patients on peritoneal dialysis. Only activity in the HPLC fraction at 19.5 ± 0.05 minutes was volume sensitive and only this factor's activity correlated with weight change during volume expansion, changes in blood pressure, and the serum levels of [Na,K]ATPase inhibition [23]. The location of this volume sensitive PD candidate was well resolved from regions of the HPLC chromatogram where ouabain (13.0 min) or digoxin (22.5 min) would elute if present.

Reversibility of the PD candidate's effects

Competition of the PD candidate, ouabain, bufalin, strophanthidin or digoxin with [^{125}I]-labeled digoxin for digoxin specific antibodies was assessed. Serial dilution of single preparations of the PD candidate or graded concentrations of the cardioglycosides were assayed in a digoxin radioimmunoassay. The PD candidate, as well as the cardioglycosides tested, showed a concentration-dependent reduction in the level of bound digoxin tracer. The curve for the PD candidate was similar to the standard curve of digoxin (Fig. 1A), but differed from other cardioglycosides which tended to have flatter displacement curves (Fig. 1B). Among the cardioglycosides, the least digoxin antibody cross reactivity was seen with ouabain. However, LPC require concentrations 10^{-3} mol/liter or greater to produce significant displacement of labeled digoxin from the digoxin antibody (data not shown).

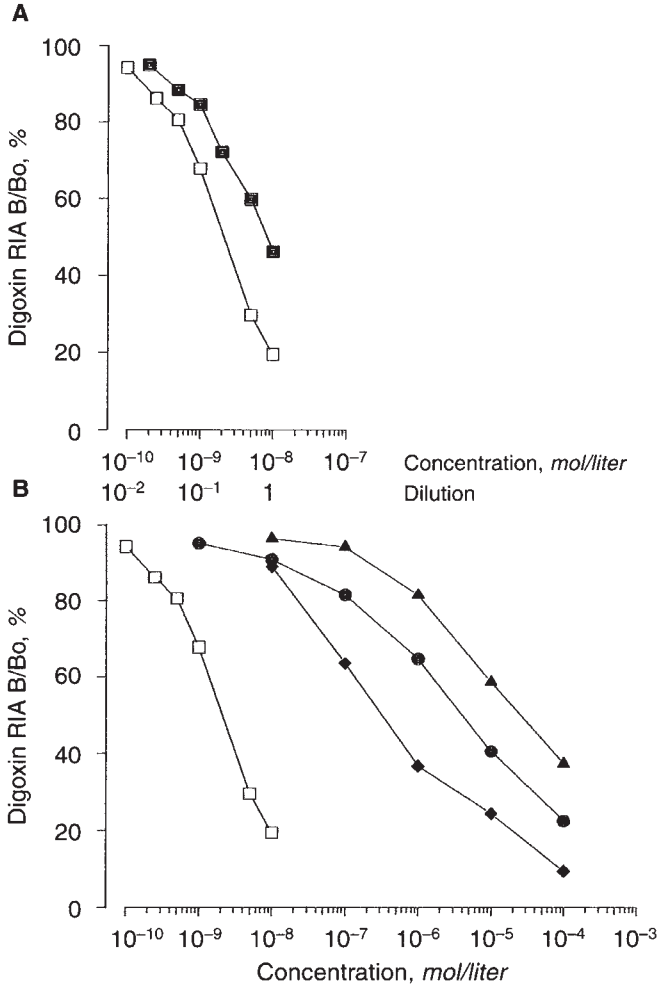

Fig. 1. *Concentration dependent digoxin RIA response of the PD candidate and cardioglycosides.* The ratio of the label bound at a given concentration of antigen (B) divided by the amount of label bound in the absence of antigen (Bo) is plotted as a function of concentration. The response to serial dilutions of a single preparation of PD candidate ("DLF", ■) or to graded concentrations of digoxin (□) is shown in **A.** Other cardioglycosides, strophanthidin (♦), bufalin (●), ouabain (▲) were tested in a similar fashion and are shown in **B.**

To provide additional evidence that the species cross reacting with the digoxin antiserum was the active inhibitory agent, other specimens of the PD candidate were divided equally and assayed both by the digoxin RIA and by inhibition of [Na,K]ATPase (Fig. 2). This comparison demonstrated a highly significant correlation between the two assays ($r = 0.95$, $P = 0.012$).

We next examined the ability of a purified Fab fragment of anti-digoxin IgG (Digibind) to prevent the effects of the PD candidate, ouabain, and bufalin. A 30 minute preincubation of the digoxin Fab with each of the three agents was found to significantly reduce their inhibition of [Na,K]ATPase hydrolysis (Table 1). Additionally, this same Fab fragment was incubated with a non-specific inhibitor, lysophosphatidyl choline, without a reduction in the LPC influence on [Na,K]ATPase activity (Table 1).

Physiologic Na pump inhibition, as opposed to non-specific enzyme inactivation or denaturation, should be reversible. Hence, evaluation of candidates should include assessment of reversibility. We exploited the PD candidate's binding to digoxin antibodies, and specifically to the digoxin Fab fragment, to probe reversibility of its influence on [Na,K]ATPase. Ouabain was used as a specific inhibitory control and LPC was used as a non-specific inhibitory control. Each inhibitor was incubated with the enzyme for 30 minutes under ionic conditions that favor tight specific binding to the digitalis receptor. This was followed with a 30 minute incubation of the inhibitor-enzyme complex with Fab fragments. As shown in Figure 3, exposure to Fab fragments after inhibitor binding to the enzyme significantly reversed the inhibition of [Na,K]ATPase activity produced by the PD candidate or ouabain, but not that produced by LPC (PD candidate, $N = 8$, from 7.8 ± 2.4 to −1.1 ± 2.0%, $P = 0.006$; ouabain, $N = 8$, from 25.4 ± 2.6 to 3.9 ± 1.2%, $P < 0.001$; LPC, $N = 6$, from 40.2 ± 1.6 to 38.1 ± 3.7%, not significant).

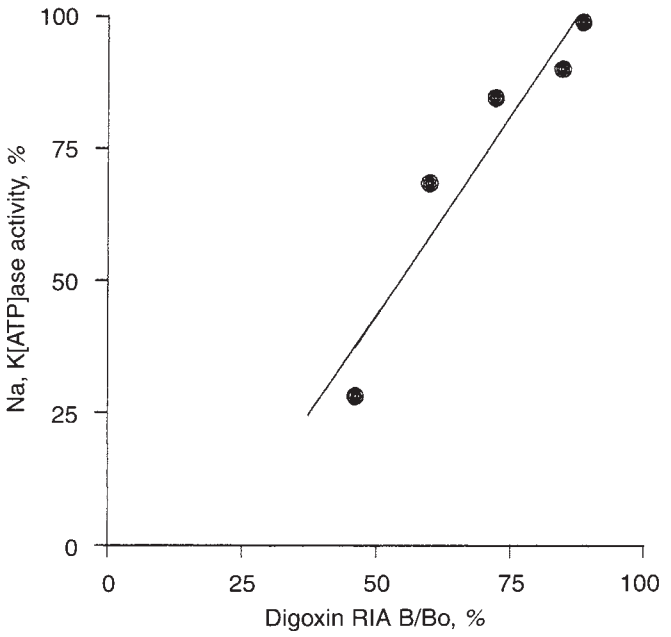

Fig. 2. *Relationship between the PD candidate's displacement of labeled digoxin from digoxin antibody and to inhibit [Na,K]ATPase activity.* Five individual purifications of the PD candidate were divided equally and assayed simultaneously in both the digoxin RIA and the [Na,K]ATPase hydrolysis assay. The line of regression is plotted. The correlation was significant ($r = 0.95$, $P = 0.012$).

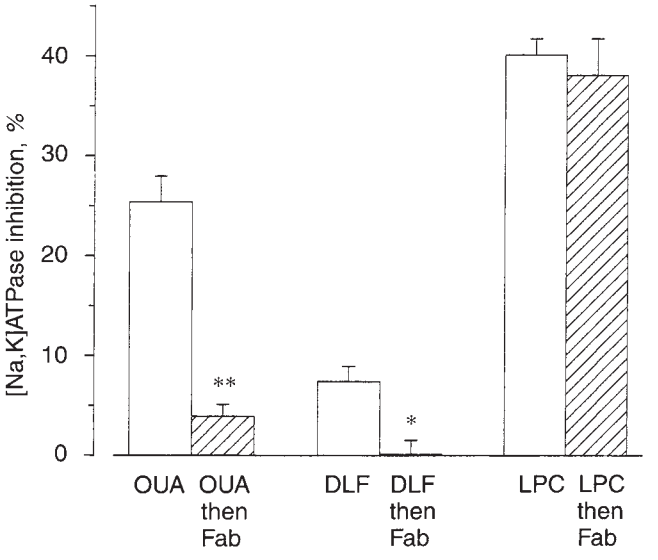

Fig. 3. *The binding affinity of Fab for ouabain and DLF was exploited to ascertain whether the inhibition induced by the various inhibitors was reversible.* Exposure to Fab reversed the inhibition induced by ouabain and the PD candidate, but did not influence the inhibition due to lysophosphatidyl choline (LPC). [Na,K]ATPase was preincubated with the PD-candidate ("DLF"), ouabain (8×10^{-8} mol/liter) or LPC (7×10^{-5} mol/liter) for 30 minutes. Half of this incubate was employed in the [Na,K]ATPase assay immediately. Half was transferred into tubes containing Fab at a final concentration of 10^{-6} mol/liter, and assayed 30 minutes later. $^{*}P < 0.01$, inhibitor versus inhibitor plus Fab; $^{**}P < 0.001$. The inhibition produced by the PD candidate alone was significantly greater than the buffer control ($P < 0.01$).

Table 1. The ability of the digoxin Fab fragment to attenuate the inhibition induced by the PD candidate, ouabain, bufalin and lysophosphatidyl choline

Agent	Inhibition of [Na,K]ATPase %		
	Sheep IgG	Dig Fab	*P*
1. PD candidate $N = 10$	18.5 ± 3.3	0.2 ± 1.4	0.0001
2. Ouabain $N = 9$	56.9 ± 7.7	3.3 ± 3.2	0.0001
3. Bufalin $N = 6$	60.9 ± 4.8	3.7 ± 2.2	0.0001
4. LPC $N = 4$	25.9 ± 2.8	29.9 ± 6.7	0.57

Paired specimens of PD candidate, ouabain (5×10^{-7} mol/liter), bufalin (1×10^{-7} mol/liter) or lysophosphatidyl choline (LPC, 7×10^{-5} mol/liter) were used. One was incubated in the presence of digoxin Fab fragments (Dig Fab, 2×10^{-6} mol/liter) and one in the presence of non-immune sheep IgG (1×10^{-6} mol/liter) prior to assessing their effects in the [Na,K]ATPase assay as described in **Methods.**

Action via the digitalis binding site of the sodium pump

We have recently demonstrated that the PD candidate produced a concentration-dependent inhibition of [Na,K]ATPase activity with mass-action kinetics appropriate for a specific inhibitor with concentration response curves that paralleled those of bufalin and ouabain [25]. Both ouabain and the PD candidate produced a concentration-dependent inhibition of labeled ouabain binding to purified [Na,K]ATPase. We then sought to test the interdependence of binding to the digitalis receptor and inhibition of hydrolytic activity. This was somewhat more complicated because the absolute concentration of the [^{3}H]ouabain solution commercially available, which was approximately 1×10^{-7} mol/liter, was sufficient to produce ~50% inhibition of [Na,K]ATPase hydrolysis. Nevertheless, using paired concentrations of ouabain of this level or greater and split specimens of the PD candidate, we measured both inhibition of [Na,K]ATPase hydrolysis and the displacement of [^{3}H]ouabain. The displacement of [^{3}H]ouabain binding by unlabeled ouabain was linearly related to the inhibition of [Na,K]ATPase activity ($r = 0.95$, $P < 0.0001$; Fig. 4). The effect of PD-DLF on labeled ouabain binding in relation to inhibition of [Na,K]ATPase activity was also highly correlated ($r = 0.91$, $P < 0.0001$). The regression line (not plotted) for the PD candidate calculated by the least squares method paralleled that of ouabain. The results suggest a specific inhibition of [Na,K]ATPase mediated by the PD candidate binding to the digitalis receptor in a manner identical to ouabain.

We studied the effect of the PD candidate and ouabain on levels of the acid-stable phosphoenzyme (E-P). Non-specific inhibitors do not increase E-P levels [28]. The PD candidate produced significant increases in both absolute and relative amounts of phosphorylated [Na,K]ATPase (Fig. 5A). Compared to the effects of ouabain, the PD candidate ($N = 5$) showed an increase in E-P levels which exceeded that produced by 10^{-6} mol/liter ouabain ($N = 6$) but was less than that produced by 10^{-5} mol/liter ouabain ($N = 4$). To clarify whether the increment of E-P caused by the PD candidate was due to increased production or to a reduction in the rate of dephosphorylation, we also studied the action of the PD candidate or ouabain on the stability of phosphoenzyme. The phosphoenzyme was incubated in a medium containing the PD candidate or ouabain with an excess of unlabeled ATP, which prevents the enzyme relabeling. As shown in Figure 5B, the

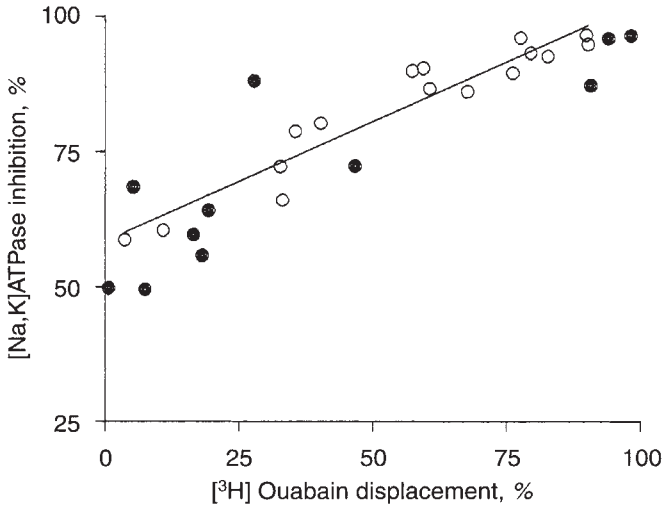

Fig. 4. *Relationship between the inhibition of [^{3}H] ouabain binding and its inhibition on [Na,K]ATPase activity.* Serial dilutions of the PD-candidate ("DLF", ●) and graded concentrations of ouabain (○) were assayed in both the ouabain binding and [Na,K]ATPase hydrolysis assays. The lowest concentration of ^{3}H-ouabain commercially available was sufficient to inhibit ~50% of hydrolysis activity. The regression line of best fit is shown for ouabain (solid line). Data points represent the average of duplicate determinations from four experiments, each employing a separate preparation of the PD candidate.

amount of E-P remaining after 10 seconds of dephosphorylation was much greater in the presence of the PD candidate or ouabain than in their absence. The increment in the level of E-P in the presence of either inhibitor was comparable to that seen in the phosphorylation experiment (Fig. 5 A, B).

Specificity of the PD candidate for [Na,K]ATPase

Whether the PD candidate's inhibition was limited to the [Na,K]ATPase, as opposed to other ATPases, was examined. This was accomplished by employing calf kidney microsomes, which have both ouabain-sensitive and -insensitive ATPase activity. The [Na,K]ATPase activity is the only ATPase activity that is ouabain sensitive. The first series of experiments, using a maximal inhibitory concentration of ouabain (10^{-3} mol/liter), determined that, on average, 35 to 40% of the total ATPase activity was ouabain-sensitive, [Na,K]ATPase activity. The combination of 10^{-3} mol/liter ouabain and 3.3×10^{-5} mol/liter thapsigargin (as an inhibitor of microsomal Ca-ATPase), induced a 69% inhibition of all ATPase activity, suggesting that much of this residual, non-ouabain inhibitable activity was Ca-ATPase. When ouabain was used at concentrations that did not produce complete inhibition of the [Na,K]ATPase, an additive effect of the PD candidate and ouabain on the ouabain-sensitive activity was found (for example, 21% inhibition for ouabain, 32% for PD-DLF and 47% for ouabain plus PD-DLF). When ouabain at a concentration that induced maximal inhibition of the ouabain-sensitive, [Na,K]-ATPase activity (1 mmol/liter) was combined with PD candidate (half of which produced significant inhibition) no further inhibition was seen. Hence, the PD candidate acts on the same fraction of ATPase activity as is ouabain-sensitive (Table 2). In contrast, oleic acid when added to 1 mmol/liter ouabain, produced signif-

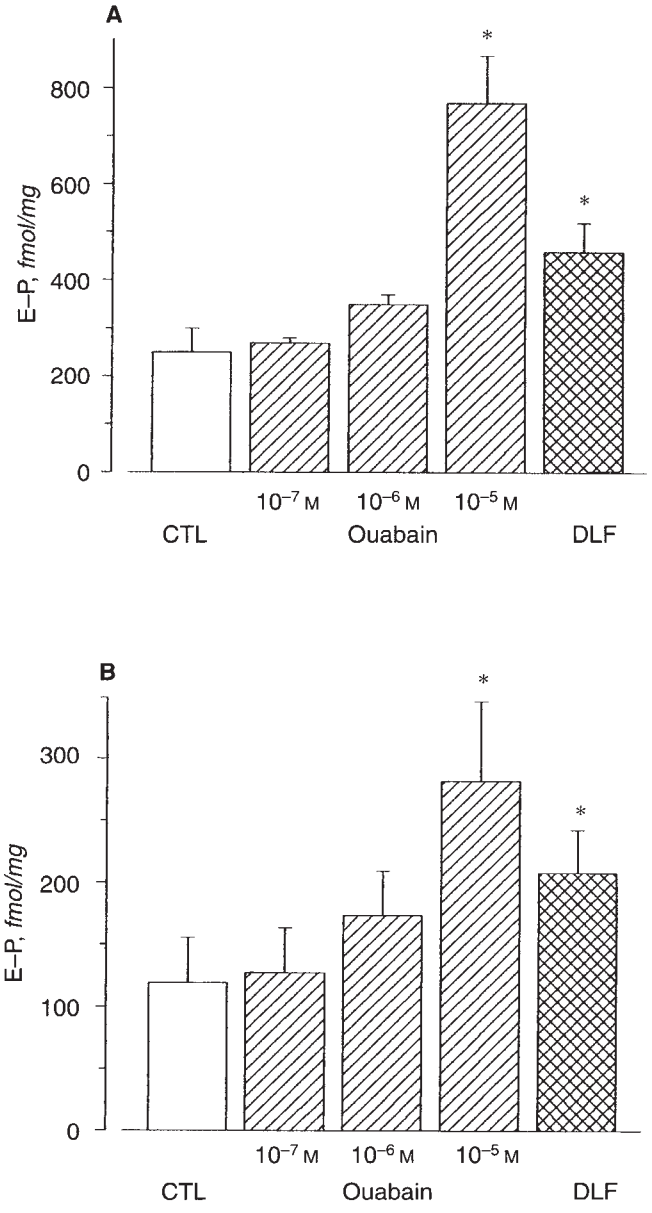

Fig. 5. *Effect of the PD candidate or ouabain on the level of acid stable phosphoenzyme (E-P).* In the first set of experiments (**A**), the level of [Na,K]ATPase phosphorylation product was measured after 20 seconds (22°C) in the presence of Mg, physiologic Na (pH 7.4) and [^{32}P]ATP and inhibitor, either PD candidate ("DLF", ▩) or ouabain (▨) at graded doses and compared to control (CTL) incubations omitting inhibitor (□). The second set of experiments measured dephosphorylation rate (**B**). After phosphorylation in the presence of Mg, Na, and [^{32}P] for 10 seconds, (4°C) dephosphorylation was initiated by adding 1×10^{-3} mol/liter cold ATP in combination with either the PD candidate (DLF) or ouabain at graded doses and allowed to continue for 10 seconds (37°C) and compared to CTL incubations omitting the inhibitor (□). An asterisk denotes a phosphoenzyme significantly greater than control ($P < 0.01$). Ouabain induced the anticipated, concentration-dependent influence of phosphorylation. The PD candidate had a similar influence, with a relative activity that lay between ouabain at 10^{-6} and 10^{-5} mol/liter. Data represent 4 to 6 determinations for ouabain and five for the PD candidate.

icantly more inhibition (44.8%) compared to maximal ouabain by itself (32.6%; Table 2).

The ionic requirement for the PD candidate inhibition of

Table 2. Specificity of the PD candidate or oleic acid for ouabain-sensitive [Na,K]ATPase

Agent	Inhibition of total ATPase activity %		
	Agent only	OUA	OUA + agent
DLF $N = 3$	18.6 ± 6.8	39.3 ± 3.7	35.7 ± 3.5
Oleic acid $N = 5$	22.7 ± 5.1	32.6 ± 1.3	44.8 ± 3.8[a]

Kidney microsomes, containing both [Na,K]ATPase and Ca-ATPase, were incubated with ouabain (OUA) at a concentration sufficient to inhibit all the [Na,K]ATPase (1 mmol/liter) or with another agent, PD-candidate (DLF) or oleic acid, as a non-specific inhibitor. At the same time we assessed the additive effects of the other half of the split PD candidate preparation or oleic acid in combination with 1 mmol/liter ouabain. [a]$P < 0.05$ for the additive effect of the agent and ouabain being greater than 1 mmol/liter ouabain alone. The PD candidate acts only on the ouabain sensitive, [Na,K]ATPase whereas oleic acid, has effects on other ATPase activity as well.

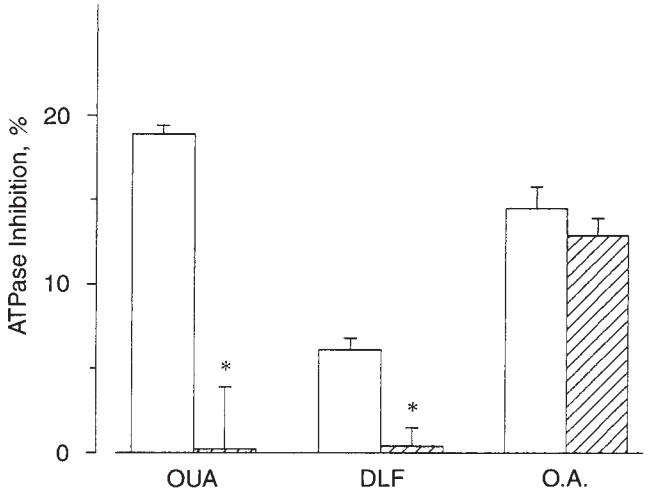

Fig. 6. *The influence of sodium and potassium on [Na,K]ATPase inhibition induced by the PD candidate, ouabain or oleic acid.* Paired specimens of ouabain (10^{-7} mol/liter), the PD candidate ("DLF"), and oleic acid (2.5×10^{-5} mol/liter) as a non-specific control were incubated with the enzyme in the presence (□) or absence (▨) of sodium plus potassium and ATP hydrolysis measured. *$P < 0.01$. The inhibition induced by PD candidate in the presence of Na and K was significant compared to omitting it ($P < 0.01$). Note that DLF shares with ouabain the requirement for sodium and potassium to induce ATPase inhibition, whereas oleic acid did not.

[Na,K]ATPase is shown in Figure 6. In the paired experiments, PD candidate produced no significant inhibition in the absence of Na and K (0.4 ± 1.1%, $N = 6$). This was significantly less than the inhibition produced by the other half of the PD candidate when tested in the same medium containing Na and K (6.0 ± 1.6, $P < 0.001$). This pattern was equivalent to that found for 1×10^{-7} mol/liter ouabain (0.0 ± 3.7 vs. 18.9 ± 0.5%, $P < 0.01$, $N = 6$). Oleic acid (2.5×10^{-5} mol/liter) produced significant inhibition in the absence of Na and K (12.9 ± 1.0%, $N = 6$), and the presence of Na and K in the medium did not alter the level of inhibition it produced (14.5 ± 1.3%, $P = 0.40$).

Discussion

Hypertension in end-stage renal disease is common and influenced substantially by volume status [1, 2]. The agent in this study is found in the peritoneal dialysate in concentrations adequate for measurement only when the patient is volume expanded, a criterion we consider crucial for candidacy [20]. One of the major problems in this field has been the large number of agents suggested as candidates based not only on a minimal number of criteria, but often on criteria that were rather nonspecific [19–22]. Because no one test is definitive for a true sodium pump inhibitor, this study employed a range of criteria to examine the specificity of the PD candidate [23]. First, because several inhibitors appear to act by a nonspecific denaturing or "detergent" effect, we examined the reversibility of the PD candidate's action. To do so, we exploited the high binding affinity of the PD candidate and other cardioglycosides for an Fab developed for digoxin. The PD candidate caused an inhibition of [Na,K]ATPase that was fully reversible following immunoneutralization with the Fab. Likewise there was strong concordance between the PD candidate's affinity for a digoxin antiserum and functional activity. Our second goal was to establish whether or not the PD candidate acted specifically at the digitalis-binding site. The PD candidate was shown to compete with labeled ouabain for the digitalis binding site, and that pump inhibition was highly correlated with this binding. We also examined the influence of the PD candidate on the phosphorylation-dephosphorylation step of the Na pump cycle. Digitalis glycosides inhibit the pump's dephosphorylation step, allowing accumulation of the phosphorylated enzyme. In this study, that action was confirmed and extended to the PD candidate, whose activity exceeded that of ouabain at 10^{-6} mol/liter, but was less active than ouabain at 10^{-5} mol/liter. Finally, we sought to establish that the PD candidate's influence was limited to [Na,K]-ATPase. We found that sodium and potassium were required for the PD candidate to cause inhibition, and it demonstrated no influence on other ATPase activity. As non-specific controls, we employed agents suggested earlier as possible candidates, oleic acid and LPC. As anticipated, both agents failed to satisfy these criteria.

Another suggested candidate, ouabain, was also examined in this study. Evidence in this study and elsewhere [23] indicates that the PD candidate differs chemically from ouabain.

Multiple regions of [Na,K]ATPase inhibition were observed in the concentrated HPLC eluate of PD [23]. For this reason, we developed a clinical protocol in which patients gradually expanded their extracellular volume and concomitantly their blood pressure. By comparing the level of sodium pump inhibition in each HPLC fraction before, during and after volume expansion, we were able to identify a single HPLC fraction that correlated closely with the volume shift. This same HPLC fraction was also the only region that was correlated with serum [Na,K]ATPase inhibitory activity and to the patient's increase in blood pressure. As such the first criterion for candidacy–a clinical or physiological correlation—was satisfied [20]. This volume sensitive factor possessed a consistent retention time on HPLC distinct from cardioglycosides and other candidates and was termed the "PD candidate" to avoid confusion.

We and others [26, 27] have used digoxin antisera to study digoxin-like factors on the premise that a digoxin antibody may recognize the endogenous ligand if structurally similar. The specificity of commercial digoxin antisera is very high for digoxin, modest for other cardiac glycosides, and low for steroid hormones and other compounds. Therefore, a digoxin radioimmunoassay might prove useful in the search for the endogenous inhibitor. The

PD candidate competed with labeled digoxin for the digoxin antibodies with a concentration dependence that paralleled digoxin. Other cardioglycosides (ouabain, strophanthidin, and bufalin) demonstrated a concentration-dependent displacement of labeled digoxin from the antibody with a slope that differed significantly from the digoxin or the PD candidate. These experiments also revealed that the amount of the PD candidate causing a 50% displacement of labeled tracer from digoxin antibody produced 57% inhibition of [Na,K]ATPase, whereas a concentration of ouabain causing 50% displacement of digoxin tracer caused more than 95% inhibition (data not shown), suggesting a much higher cross reactivity for the PD candidate than ouabain, and perhaps also explaining the different response curves. Digoxin antisera do not measure functional activity, and since some researchers had found no association between digoxin-like immunoreactivity and inhibition of [Na,K]ATPase [14], the digoxin-like cross reactivity of the PD candidate was compared to [Na,K]-ATPase inhibition. This revealed a marked correlation, indicating the inhibition was proportional to its cross reactivity, and suggested strongly that the PD candidate, an active inhibitor, is also recognized by the antibody. Because it was possible that two agents co-eluted on HPLC, each active in only one assay, we employed other experiments.

Having evidence for an interaction between our inhibitor and the digoxin antibody, we wanted to confirm that finding by using a digoxin antibody to interrupt the PD candidate's inhibition of [Na,K]ATPase activity. To do this we chose purified Fab fragments of a digoxin antiserum (Digibind) for two reasons. First, the absence of other serum components ensured that interactions seen represented PD candidate binding to the Fab. Second, if the Fab produced the anticipated effect, it provided a preparation more suitable than whole anti-digoxin antiserum with which to attempt *in vivo* experiments [7]. Digoxin Fab neither influenced [Na,K]ATPase activity by itself, nor did it influence the inhibition produced by LPC, a non-specific inhibitor. In contrast, in parallel experiments, Fab fragment preincubation with the PD candidate or ouabain completely blocked their inhibition of [Na,K]ATPase, presumably by binding the inhibitors. This observation is in agreement with *in vitro* and *in vivo* studies [7, 30–32].

Having confirmed an interaction of the PD candidate with the digoxin Fab, we had a probe with which to "neutralize" the PD candidate's effects. We then demonstrated that PD candidate or ouabain-induced inhibition of [Na,K]ATPase was rapidly reversed by a 30 minute incubation with the digoxin Fab fragment. In marked contrast, LPC-induced inhibition was not affected by Fab treatment. If it is to serve as a physiological regulator, the endogenous inhibitor's action on the cardioglycocide receptor should be reversible. Digoxin Fab reversal of pre-established binding of the PD candidate to [Na,K]ATPase with consequent reversal of inhibition is consistent with such an action and our first hypothesis. A similar finding has been reported by Balzan et al, who studied a partially purified candidate from cord blood [31].

Binding of cardioglycosides to their receptor has been extensively studied and the characteristics shown to be very specific. Hence, we assumed that a candidate's response to binding conditions should be identical to the cardioglycosides if it occupies the same site. Recently we demonstrated that the PD candidate binding and subsequent inhibition were amplified by a pre-reaction environment high in Mg and Pi, responses limited to cardioglycosides [24, 25], providing support for an action at the same binding site.

The PD candidate reduced [^{3}H]ouabain binding to its receptor in a concentration dependent manner, and inhibition of [Na,K]-ATPase hydrolysis was highly correlated to this binding, in parallel with ouabain. This provided additional evidence that the PD candidate and ouabain share the same binding site and that [Na,K]ATPase inhibition depends on this binding. However neither of these approaches examines the mechanism of inhibition. A rigorous criterion demanded that we establish that the PD candidate induced inhibition at the same state of the enzyme cycle as known cardioglycosides.

During the Na pump cycle, the enzyme is phosphorylated by ATP in the presence of Mg and Na and then dephosphorylated in the presence of K. *In vitro*, the isolated enzyme can also be phosphorylated in reverse, starting with Mg+Pi. The phosphorylation of [Na,K]ATPase from Pi in the presence of Mg is increased by cardioglycosides by way of a mechanism that involves reduced dephosphorylation [33, 34]. The concentration dependence of Na pump inhibitors on phosphoenzyme level and on inhibition of [Na,K]ATPase activity is roughly the same [34]. This promotion of ^{32}P incorporation from Pi into [Na,K]ATPase has been suggested to be the most rigorous criterion for classifying a compound as truly digitalis-like, and that approach has been confirmed [19, 28]. The influence of cardioglycosides on the phosphoenzyme level in the presence of Na, Mg and ATP is more complex. Some studies report enzyme phosphorylation from ATP being completely prevented by ouabain [36, 37], while others showed no ouabain effect [38]. The available data suggests that ouabain's effect on phosphorylation varies with temperature, ATP concentration, sodium concentration and the ionic strength of the medium [39, 40]. In this study the PD candidate, like ouabain, produced a significantly increased level of phosphoenzyme in the presence of Mg, Na and ATP, suggesting that it acts via the same binding site and mechanism. The dephosphorylation rate of phosphoenzyme was also measured. As expected, the PD candidate increased phosphoenzyme as a result of a reduction of the dephosphorylation rate, as seen with cardioglycosides [34, 40, 41]. The ability of the PD candidate, like ouabain, to stabilize phosphoenzyme, not only from Pi but also from ATP, suggests that specific inhibition of [Na,K]ATPase is due primarily to a prolonged half life of the enzyme-inhibitor complex, which cannot be rephosphorylated by ATP, and thereby interrupts the pump cycle. The exacting constraints of these observations support the contention that this approach discriminates true candidates from other [Na,K]ATPase inhibitors [19, 28], and the findings conform well with our second hypothesis.

To determine whether the PD candidate produced inhibition limited to the [Na,K]ATPase and does not extend to other ATPases, we took advantage of an unpublished observation: Bovine renal microsomal ATPase activity was only partially inhibited by ouabain (35 to 45% of total activity) at a concentration sufficient to inhibit all [Na,K]ATPase, suggesting the presense of ATPase activity unrelated to the sodium pump. In agreement, thapsigargin, a specific inhibitor of intracellular Ca-ATPase [29], blocked 47% of total ATPase activity, and when ouabain and thapsigargin were used together, a marked additive inhibition was measured (69% of total). Therefore, both [Na,K]ATPase and Ca-ATPase were present. Oleic acid, a non-specific inhibitor, produced an inhibition of ATPase which significantly exceeded

that seen with high ouabain concentrations, that is, it inhibited both the [Na,K]ATPase and other ATPases. The PD candidate inhibited [Na,K]ATPase, and its effects were additive to those of ouabain, when ouabain was present at a submaximal concentration. However, the PD candidate did not effect ouabain-insensitive ATPase activity, that is, it did not produce further inhibition when added to a maximal inhibitory concentration of ouabain. While these experiments were consistent with our hypothesis, the heterogeneity of the microsome preparation might be open to other interpretation. Therefore, in a second series of experiments we assessed the Na and K dependence of the PD candidate's activity. Again oleic acid, as a non-specific inhibitor, produced an inhibition of ATPase activity that did not require Na and K, whereas both ouabain and the PD candidate produced inhibition only in the presence of Na and K. Together these two experiments support our third hypothesis.

Although the PD candidate has an action on the sodium pump indistinguishable from all known cardioglycosides, it displays a number of physical-chemical differences. Perhaps the most striking and important is its chemical lability [42]. While many compounds may enter the organism through the diet and accumulate in the patient with renal failure, the marked lability of the PD candidate makes this unlikely. In addition, we controlled diet over the course of the study. Only fluid and salt intake were modified. In addition, the HPLC gradient employed in these experiments separates our factor cleanly from most other candidates, including ouabain, digoxin, bufalin, and the common steroid hormones [43]. Finally, when we employed an antiserum directed against digoxin, the interaction of the PD candidate and the antibody resembled that of digoxin far more closely than did not only ouabain, but also strophanthidin and bufalin.

In summary, these experiments provide consistent and compelling evidence that the labile PD candidate, shown previously to be correlated with volume status, blood pressure and serum levels of [Na,K]ATPase inhibition, also acts specifically on the sodium pump, binding to the digitalis-binding site, and reversibly inhibiting the sodium pump cycle in a manner identical to known cardioglycosides. Thus, it fulfills important and rigorous criteria for candidacy as an endogenous sodium pump inhibitor.

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